IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	)	Examiner: CHUNDURU, S.
	)	
Roland Carlsson et al.	)	Art Unit: 1637
	)	
Serial No.: 09/734,801	)	Response to Paper No. 16
	)	
Filed: December 12, 2000	)	
	)	
For: "A METHOD FOR IN VITRO MOLECULAR REVOLUTION OF PROTEIN FUNCTION"	)	

DECLARATION OF PROFESSOR MATHIAS UHLEN

I, Mathias Uhlen, hereby declare that:

1. I am a Professor at the Department of Biotechnology, SCFAB, Royal Institute of Technology (KTH), Stockholm, Sweden. I have over 20 years of scientific training and research experience in molecular biology and recombinant DNA technology. I have published more than 250 scientific publications. I am an inventor or co-inventor of more than 50 international patent applications. I have been a member of organizing committee for more than 20 international conferences. I also sit on numerous Boards of Directors, including the Board of Directors of Amersham plc and Pyrosequencing AB. Additional details of my credentials are set forth in my *Curriculum vitae*, attached hereto.

2. I have read and am familiar with U.S. Patent Application No. 09/734,801, entitled: "A Method for *in vitro* Molecular Evolution of Protein Function", (hereinafter '801 application). I have also studied the Official Action dated December 17, 2002 in the '801 application, as well as the disclosure of Kikuchi et al. (*Gene*, Vol. 243, pp. 133-137, 2000) and U.S. Patent No. 6,159,690 (hereinafter '690 patent) cited by the Examiner. As a scientist having considerable current knowledge and experience in the field of the invention of the '801 application, I do not concur with the Examiner's position, as set forth in the December 17, 2002 Official Action, that claims 2-6 of '801 application are obvious over Kikuchi et al. and '690 patent.

3. I have also reviewed the Terminal Disclaimer submitted

concurrently. I understand that by such a Terminal Disclaimer, '690 is no longer a citable reference for '801 application.

4. Claims 2-6 of '801 application are directed to a method for generating a polynucleotide sequence(s) from single-stranded parent polynucleotide sequences, which method comprises a) digesting single-stranded parent polynucleotide sequences with an exonuclease; b) allowing the resultant single-stranded DNA fragments to anneal to each other; and c) amplifying the annealed DNA fragments, wherein, the exonuclease is BAL31 or the parent polynucleotide sequences have been subjected to mutagenesis.

5. Kikuchi et al. discloses a method for shuffling DNA comprising digesting single-stranded DNA with DNase I and allowing the DNA fragments formed thereby to anneal to each other.

6. However, contrast to the Examiner's assertion, at page 5 of the Official Action, that Kikuchi et al. teaches "digesting each single stranded polynucleotide sequences with an exonuclease to generate a first population of single stranded fragments (plus strand) and a second population of single stranded fragments (minus strands)", it is noted that the nuclease taught by Kikuchi et al. to digest single stranded DNA is DNase I, an endonuclease, not an exonuclease.

7. Kikuchi et al. makes no mention of any problems or disadvantages associated with the use of DNase I. Indeed, Kikuchi et al. does not comment on the importance or otherwise of the nuclease used to digest the starting material. Rather, the key teaching of the Kikuchi et al. is the use of single-stranded DNA.

8. I believe that DNase I would be used as the nuclease in the initial digestion step because the Kikuchi et al. has already demonstrated that this nuclease can be used effectively in such methods. Moreover, the Kikuchi et al. presents DNase I as having advantageous properties (see Introduction):

"We took advantage of DNase I to cleave ssDNA ..."

This would suggest to a skilled person that if one didn't use DNase I there

was a risk that the advantageous properties of DNase I might be lost.

9. Hence, I see no reason why a skilled person seeking to use the methods described in Kikuchi et al. to produce variant polynucleotides would choose to alter the initial digestion step by using a nuclease other than DNase I.

10. Additionally, I have reviewed the examples in '801 application and the further experimental evidence of the improved control of fragment size attainable by digesting with an exonuclease using the methods of '801 application (see Appendix 2).

11. In my view, such advantageous effects could not have been predicted by a skilled person on the basis of Kikuchi et al. Accordingly, claims 2-6 of '801 application are not obvious over Kikuchi et al.

12. In summary, it is my opinion that a skilled person working in the field of recombinant DNA technology would not have tried the methods disclosed in '801 application after reading Kikuchi et al.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so make are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued thereon.

May 02, 2003
DATE

Mathias Uhlen

Curriculum vitae - Mathias Uhlén

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Education/exams/employments

Chemical Engineer, Royal Institute of Technology, 1979
Doctor of Technology, Royal Institute of Technology (KTH), Stockholm, 1984.
Postdoctoral position, EMBL, Heidelberg, Germany (1985-86)
Professor, Biotechnology, KTH, Stockholm (1988-)
Vice-President external affairs, KTH, Stockholm (1999-2001)

Experience

Approximately 250 full-length scientific publications
More than 50 international patent applications
Member of organising committee of more than 20 international conferences
Invited speaker to more than 100 international conferences
50 PhDs have been supervised or co-supervised to their dissertation

Professional service

Member of the Royal Swedish Academy of Science (KVA), 1993-
Member of the Swedish Technical Research Council (TFR), 1991-1998
Member of the Swedish Academy of Engineering Science (IVA), 1992-
Member of HUGO (Human Genome Organization), 1993-
Member of European Molecular Biology Organization (EMBO), 1995-
Chairman of the National committee for Biochem. and Mol. Biology 1994-1999
Director for SSF National Grad. School Cell Factory for Funct. Genomics 1998-2002
Advisory Board for Genethon Genome Center, France, 1990-1994

Member of Forskningberedningen (advisor to the Ministry of Education), 1999-2002

Advisory Committee for Centre National de Genotypage, France, 2000-

Advisory Committee for the German Resource Center for Genomics, 2000-

Honors and awards

Alfred and Hilda Eriksson Prize, 1989, the Royal Swedish Academy of Science.

The Göran Gustafsson Prize, 1994, the Royal Swedish Academy of Science.

The Pierce Award, 1995

Industrial experiences

Member of the Board of Directors:

Pharmacia Biotech (Uppsala), 1992-1997

Fermentech Inc, Edinbourgh, Scotland 1995-2000

KTH School of Industrial Management 1996-1998

Stockholm Science Park (Teknikhöjden AB) 1996-2001

Amersham Pharmacia Biotech (London), 1997-2001

Amersham Biosiences Inc (New Jersey, USA), 2001-2002

Amersham Plc (London, UK), 2002-

Pharmacia and Upjohn Diagnostics AB (Uppsala) 1997-2000

KTH Holding AB (Stockholm) 1999-

KTH Näringslivskontakt AB (Stockholm) 2000-2002

KTH Executive School AB (Stockholm), 2001-

Pyrosequencing (Uppsala), 1997-

Affibody (Stockholm), 1998-

Visual Bioinformatics (Stockholm), 2000

Scandigen (Stockholm), 1992-1999

Skanditek (Stockholm) 2000-

Prevas (Stockholm), 2000-2003

Vitrolife (Göteborg), 2000-2002

Personal Chemistry (Uppsala), 2000-

Magnetic Biosolutions (Stockholm), 2000-

Creative Peptides Sweden (Stockholm), 1998-2002

Wood Heads (Umeå), 2000-2001

Appendix 1

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Education/exams/employments
Chemical Engineer, Royal Institute of Technology, 1979
Doctor of Technology, Royal Institute of Technology (KTH), Stockholm, 1984.
Postdoctoral position, EMBL, Heidelberg, Germany (1985-86)
Professor, Biotechnology, KTH, Stockholm (1988-)
Vice-President external affairs, KTH, Stockholm (1999-2001)

Experience
More than 250 full-length scientific publications
More than 50 international patent applications
Member of organising committee of more than 20 international conferences
Invited speaker to more than 100 international conferences
43 PhDs have been supervised or co-supervised to their dissertation
Member of several Board of Directors, including Amersham Biosciences and Pyrosequencing

Professional service
Member of the Royal Swedish Academy of Science (KVA), 1993-
Member of the Swedish Technical Research Council (TFR), 1991-1998
Member of the Swedish Academy of Engineering Science (IVA), 1992-
Member of HUGO (Human Genome Organization), 1993-
Member of European Molecular Biology Organization (EMBO), 1995-
Chairman of the National committee for Biochem. and Mol. Biology 1994-1999
Director for SSF National Grad. School Cell Factory for Funct. Genomics 1998-2002
Advisory Board for Genethon Genome Center, France, 1990-1994
Member of Forskningberedningen (advisor to the Ministry of Education), 1999-
Advisory Committee for Centre National de Genotypage, France, 2000-
Advisory Committee for the German Resource Center for Genomics, 2000-

Honors and awards
Alfred and Hilda Eriksson Prize, 1989, the Royal Swedish Academy of Science.
The Göran Gustafsson Prize, 1994, the Royal Swedish Academy of Science.
The Pierce Award, 1995

APPENDIX 2

Improved control of fragment size using exonucleases

(A) Exonucleases

The following examples demonstrate how exonuclease digestion allows the creation of fragments of various and controllable sizes depending on the conditions used.

BAL 31

Single-stranded DNA was digested with BAL31 according to the protocol in Example 1, with an enzyme concentration of 4.2 U/ml reaction volume and ssDNA concentration of 0.008 µg/µl reaction volume.

In a typical experiment, about 300 ng DNA is isolated at each time point of BAL31 treatment. Figure 1 shows an agarose electrophoresis gel image of such an experiment with untreated ssDNA in lane 4 and ssDNA treated for 10 minutes in lane 3, for 30 minutes in lane 2 and for 50 minutes in lane 1. Lane 5 is the molecular weight (MW) standard.

Figures 2 to 4 shows the corresponding gel chromatograms of the lanes, respectively. Figure 2 is the untreated material and the multiple peaks refer to different conformations of the ssDNA. Figure ~~14~~ 3 corresponds to lane 3 and material treated for 10 minutes. The material was heat treated to stop the enzymatic reaction, and thus resolving the different conformations, and one peak of a distinct size is shown. Figure 4 corresponds to lane 2 and material treated for 30 minutes.

Here it is clear that the peak corresponding to larger fragments is decreasing and a peak of smaller DNA fragments has appeared.

Exonuclease VII

Single-stranded DNA was digested with exonuclease VII using an enzyme concentration of 7.7 U/ml reaction volume and ssDNA concentration of 0.008 µg/µl reaction volume. The reaction buffer comprised 67 mM potassium phosphate (pH 7.9), 10 mM mercaptoethanol, 6.7 mM $MgCl_2$ and 8.3 mM EDTA.

The reaction was allowed to proceed at 37°C for 10, 20 and 30 minutes, before being stopped by heat inactivation (95°C for 5 minutes).

In Figure 5 the fragmentation pattern using exonuclease VII is shown. Lane 1 is MW standard, lane 2 is untreated ssDNA, lane 3 is ssDNA fragmented with exonuclease VII for 10 minutes, lane 4 is ssDNA fragmented with exonuclease VII for 20 minutes, and lane 5 is ssDNA fragmented with exonuclease VII for 30 minutes. This shows that the fragment sizes are decreased by time.

Exonuclease Rec J_f

Single-stranded DNA was digested with exonuclease Rec J_f using an enzyme concentration of either 2.5 U/ml reaction volume or 10 U/ml reaction volume and ssDNA at a concentration of 0.007 µg/µl reaction volume, corresponding to 0.36 U enzyme/µg DNA and 1.4 U enzyme/µg DNA, respectively. The reaction buffer comprised 50 mM NaCl, 10 mM Tris.HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol, at pH 7.9

The reaction was allowed to proceed at 37°C for 10, 20 and 30 minutes, before being stopped by heat inactivation (95°C for 5 minutes).

In Figure 6 the fragmentation pattern using exonuclease Rec J_f at 036 U/microgram ssDNA is shown. Lane 1 untreated ssDNA, lane 2 is ssDNA fragmented with exonuclease Rec J_f for 10 minutes, lane 3 is ssDNA fragmented with exonuclease Rec J_f for 20 minutes, and lane 4 is ssDNA fragmented with exonuclease Rec J_f for 30 minutes. This shows that the fragment sizes are decreased by time. In Figure 7 the enzyme concentration is increased 4 times (1.4 U/microgram ssDNA) and the fragmentation pattern is shown from 0 to 30 minutes, showing a higher degree of fragmentation as compared to Figure 6. This shows that both time and enzyme concentration can be used to control the fragmentation.

(B) Endonucleases

Conventional DNA shuffling methods typically use DNase I for fragmentation (for example, see Stemmer, 1994, *Nature* **370**:389-391). DNase I cleaves DNA in an endonucleolytic fashion at sites adjacent to pyrimidines. Consequently, not all possible fragment sizes can be obtained.

Moreover, using magnesium in the reaction buffer, a homologous mix of mono- and oligomers is obtained. Hence, different methods such as gel agarose electrophoresis purification or gel filtration need to be used in order to isolate fragments of different sizes. Often fragments of small size or a mix of small and larger fragments are desired to optimise recombination. However, these purification methods introduce single-stranded nicks in the double-stranded PCR products. Fragments of a particular size purified on a gel would thus consist of dsDNA with a large number of single-stranded nicks, which would give rise to many smaller fragments upon denaturation. This means that many of the single-stranded fragments generated upon

denaturation would be too short to function as primers during the annealing, resulting in a great loss of product.

Using manganese in the reaction buffer creates fragments of sizes smaller than 50 bp and no gel purification is needed. However, here you are restricted to use only small fragments and these can not be mixed with larger fragments, something that would probably increase the recombination frequency.

The problems associated with the use of endonucleases are demonstrated in the following experiments:

DNase I

DNA was digested for 5 minutes with DNase I at a concentration of 0.15 U/μg DNA.

Magnesium and manganese buffers were compared when fragmenting with DNase I and the result is shown in Figure 8. Lane 1 is MW standard, lane 2 is untreated ssDNA in Mg buffer, lane 3 is ssDNA fragmented with DNase I in Mg buffer according to Stemmer (1994) *Nature* **370**:389-391, lane 4 is untreated ssDNA in Mn buffer and lane 5 is ssDNA fragmented with DNase I in Mn buffer according to Kikuchi *et al.* (2000) *Gene* **243**:133-137. It is clear from Figure 8 that, when using Mg buffer and conditions according to the Stemmer and Kikuchi papers, no fragmentation occurs. Moreover, when using Mn buffer and conditions according to the Stemmer and Kikuchi papers, all material is totally fragmented within only a few minutes.

In an attempt to obtain fragments of different sizes we decided to use Mg buffer and increase the enzyme concentration. Figure 9 shows an agarose electrophoresis gel image of such an experiment using DNase I. Lane 1 is

the MW standard. Lane 6 is untreated ssDNA. Lane 12 is ssDNA treated according to the Stemmer and Kikuchi papers, using 0.15 U enzyme/microgram DNA and lane 13 is the same material treated with 1 U enzyme/microgram DNA (i.e. six times more enzyme).

Figures 10 to 12 shows the corresponding chromatograms. The untreated ssDNA has been heat treated, therefore only one peak appears in Figure 10 (indicated by arrow). In Figure 11, it is apparent that using the amount of DNase I according to the Stemmer and Kikuchi papers the peak for untreated ssDNA is somewhat decreased (indicated by arrow) but no distinct peak is visible for the fragmented DNA, only a smear. Using 6 times more enzyme the untreated ssDNA is totally abolished (Figure 12) and neither here is any visible peak of the fragments.

Mung bean nuclease

Single-stranded DNA was digested with Mung bean nuclease (Product No MO250S, New England Biolabs) using an enzyme concentration of either 0.375 U/ml reaction volume and ssDNA at a concentration of 0.007 μg/μl reaction volume. The reaction buffer comprised 50 mM sodium acetate, 30 mM NaCL, 1 mM $ZnSO_4$, at pH 5.0.

The reaction was allowed to proceed at 25°C for 10 minutes, before being stopped by heat inactivation (95°C for 5 minutes).

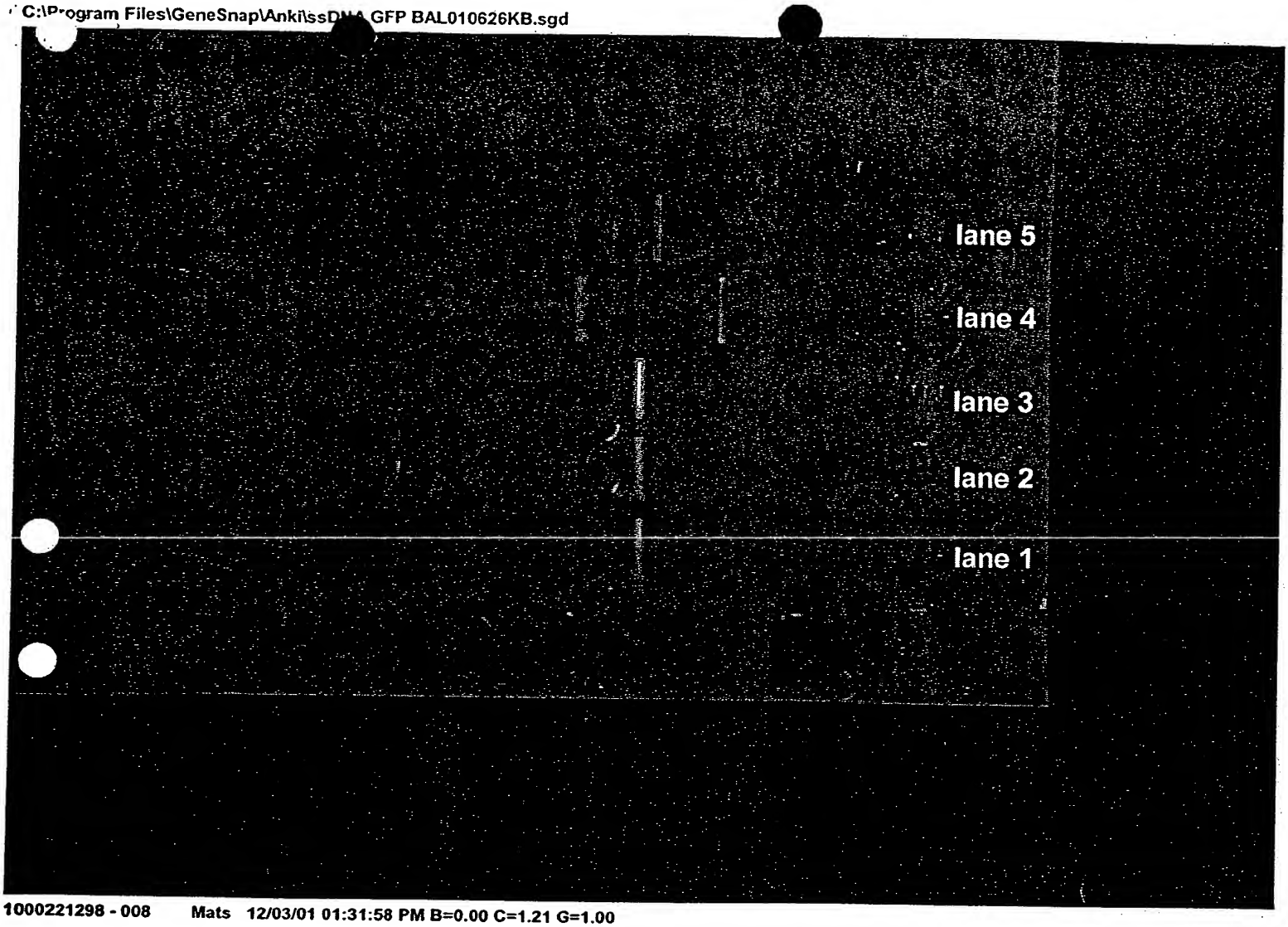
Figure 13 shows fragmentation using another endonuclease, Mung bean nuclease. Lane 1 is the untreated ssDNA, lane 2 is the same material treated for 10 minutes. Lane 3 is the MW standard.

Results indicate that all DNA was totally fragmented after only 10 minutes digestion with Mung bean nuclease (see lane 2), despite using the enzyme at a concentration lower than that recommended by the manufacturer.

Conclusions and comments

The above examples show how the fragment sizes can be controlled using exonucleases and altering the reaction conditions, *i.e.* time, reaction volume, enzyme concentration. The different peaks are visualised using gel image chromatograms.

In contrast, using endonucleases, such as DNase I, gives a reaction which is hard to control. Using conditions as referred in the literature, either using Mg or Mn containing buffers, typically gives a situation when either everything or nothing is fragmented. An experiment using another endonuclease (Mung bean nuclease) confirms these observations.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lane 5
lane 4
lane 3
lane 2
lane 1
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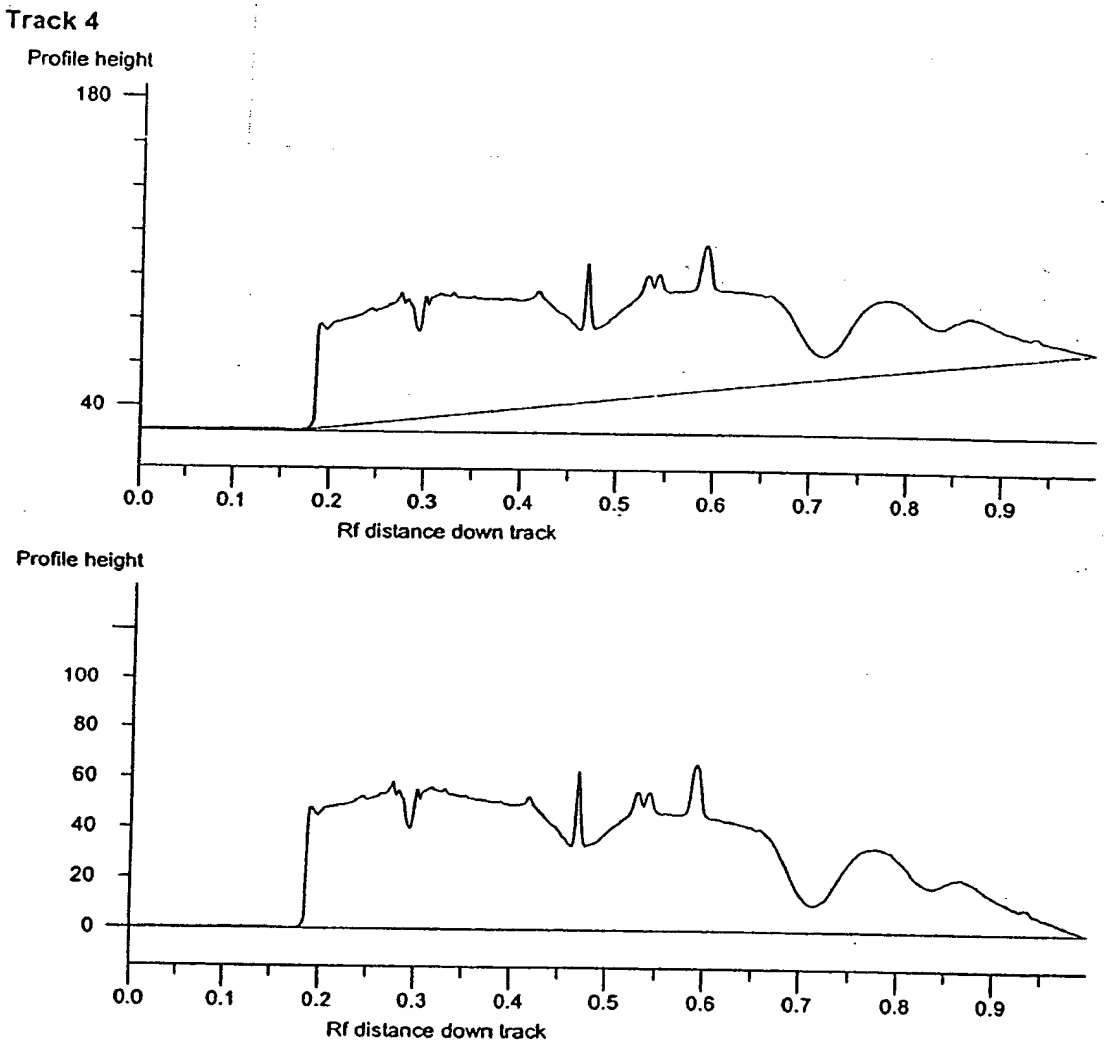
FIGURE 1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0.2
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0.6
0.7
0.8
0.9
Rf distance down track
Profile height
100
80
60
40
20
0
0.0
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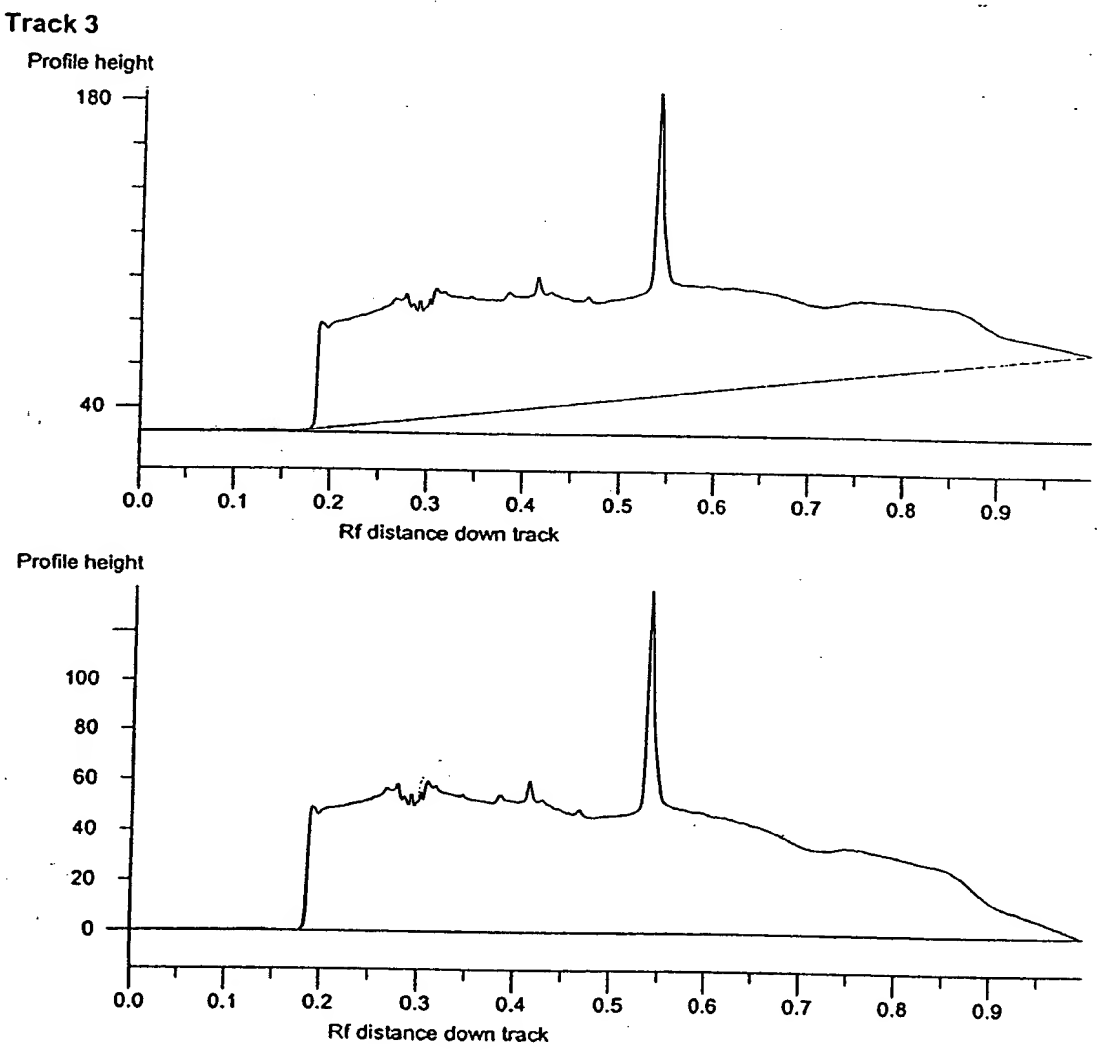
0.5
0.6
0.7
0.8
0.9
Rf distance down track

FIGURE 2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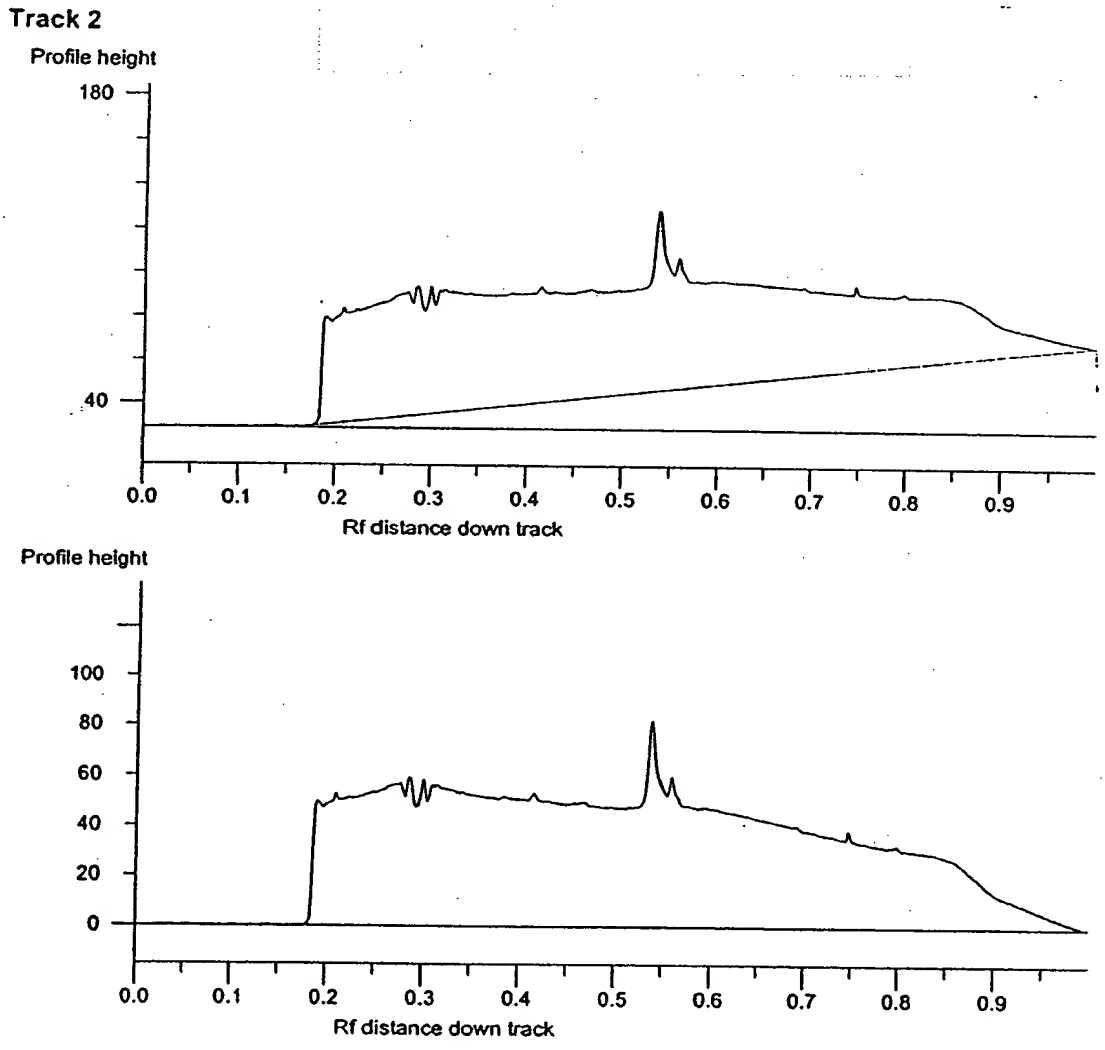
0.4
0.5
0.6
0.7
0.8
0.9
Rf distance down track
Profile height
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Rf distance down track

FIGURE 3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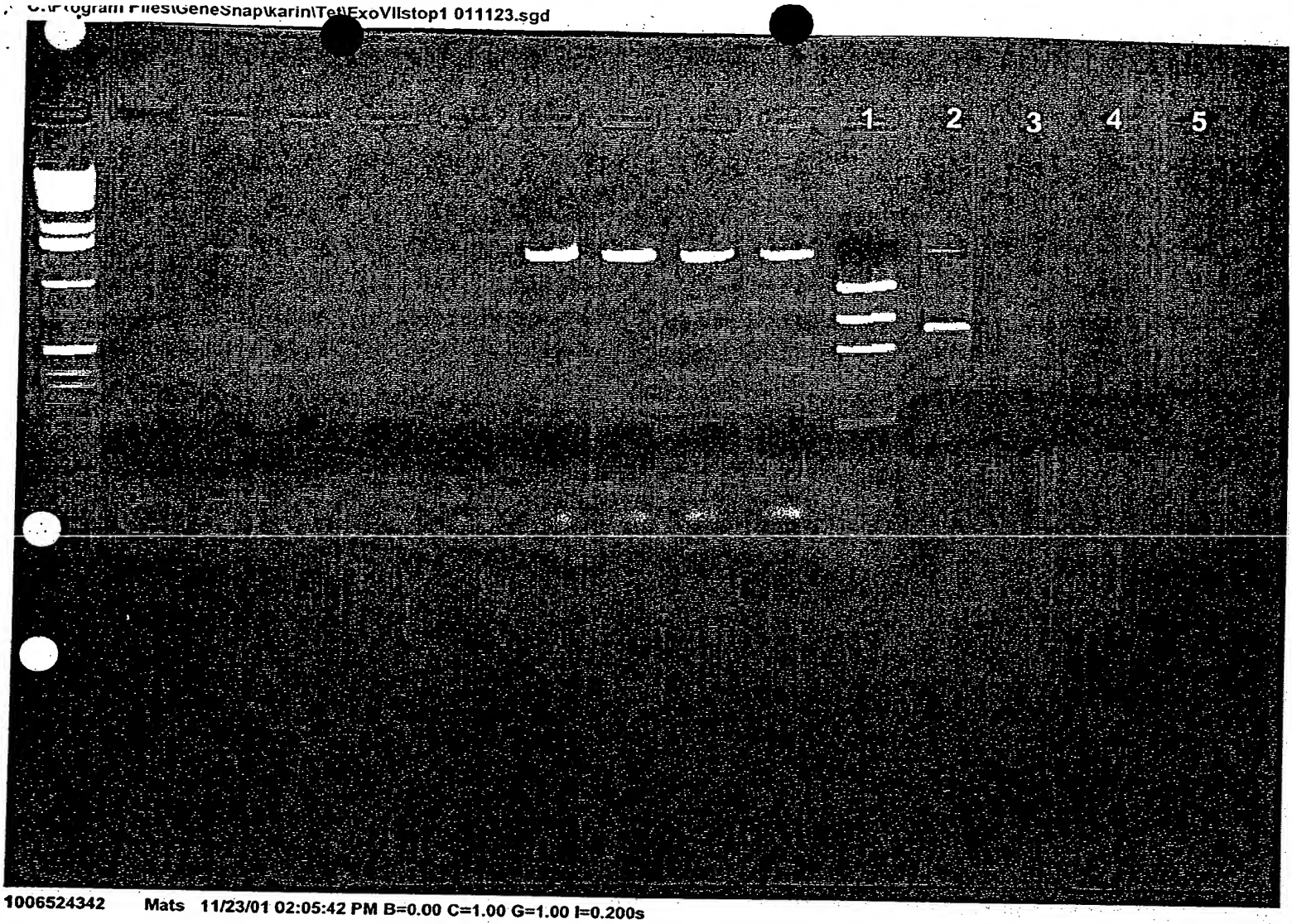
0.6
0.7
0.8
0.9
Rf distance down track
Profile height
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Rf distance down track

FIGURE 4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4
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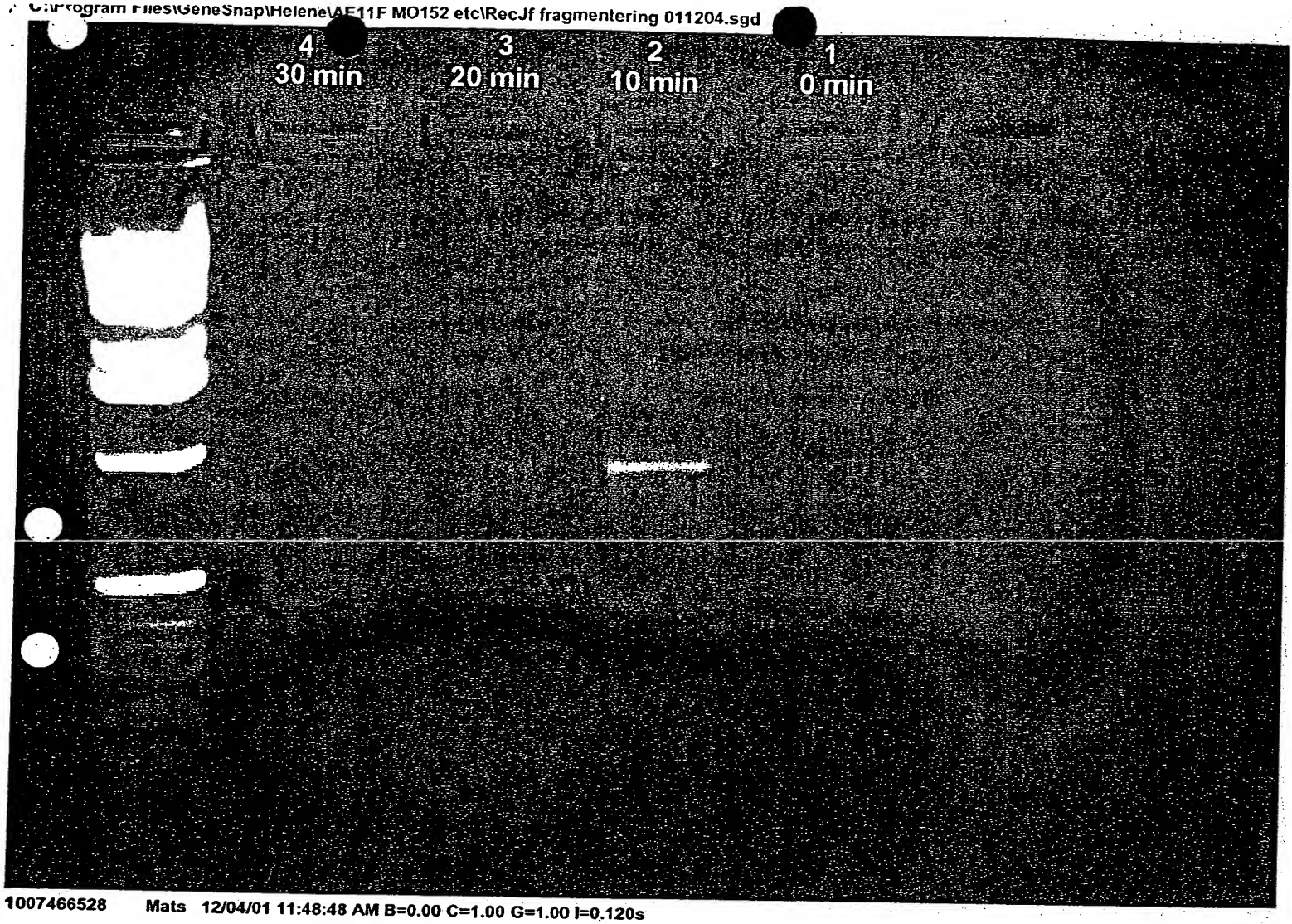
FIGURE 5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30 min
3
20 min
2
10 min
1
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1007466528 Mats 12/04/01 11:48:48 AM B=0.00 C=1.00 G=1.00 I=0.120s

FIGURE 6

C:\Program Files\GeneSnap\Helene\AE11F MO152 etc\RecJf fragmentering 011205.sgd

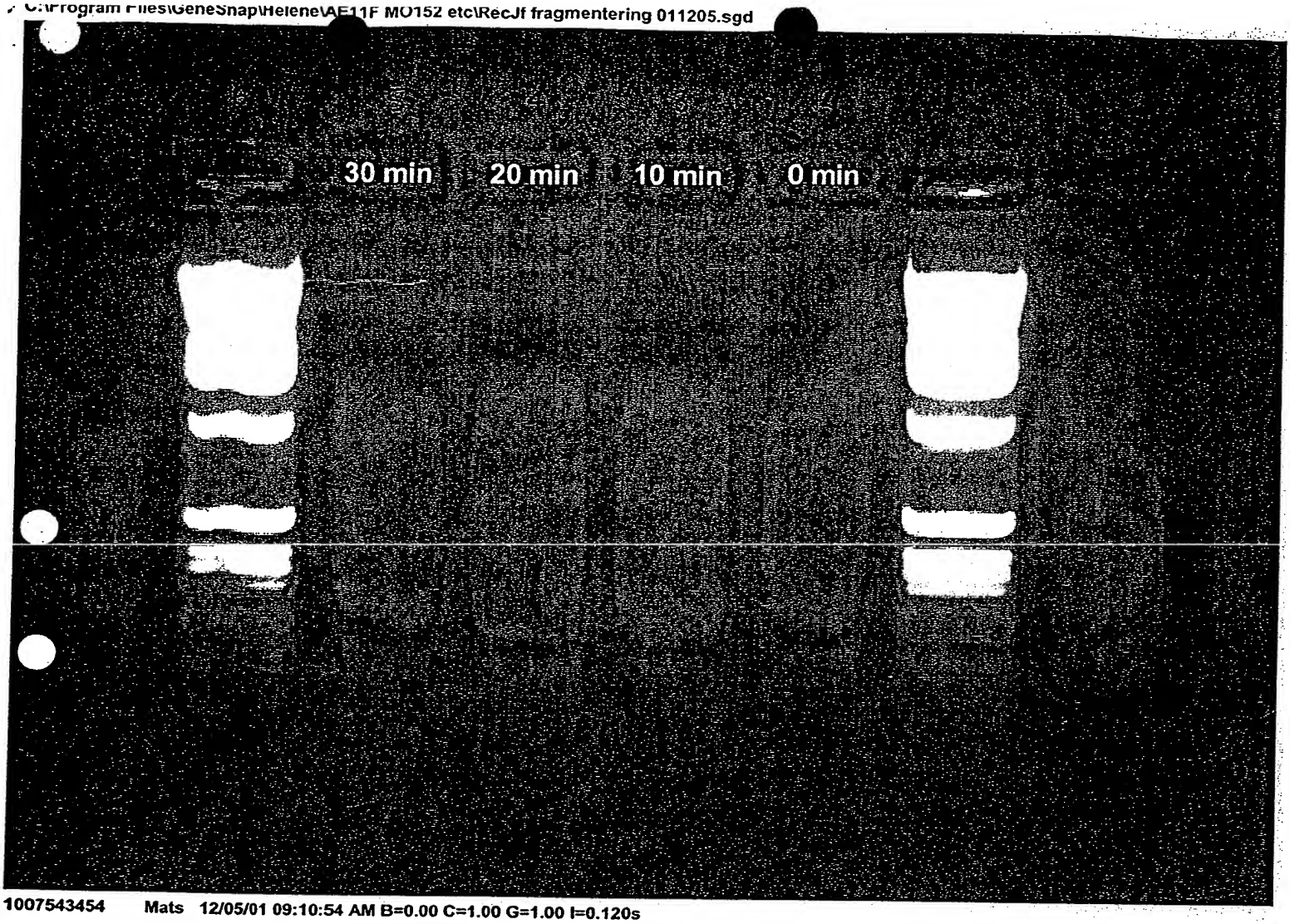

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FIGURE 7

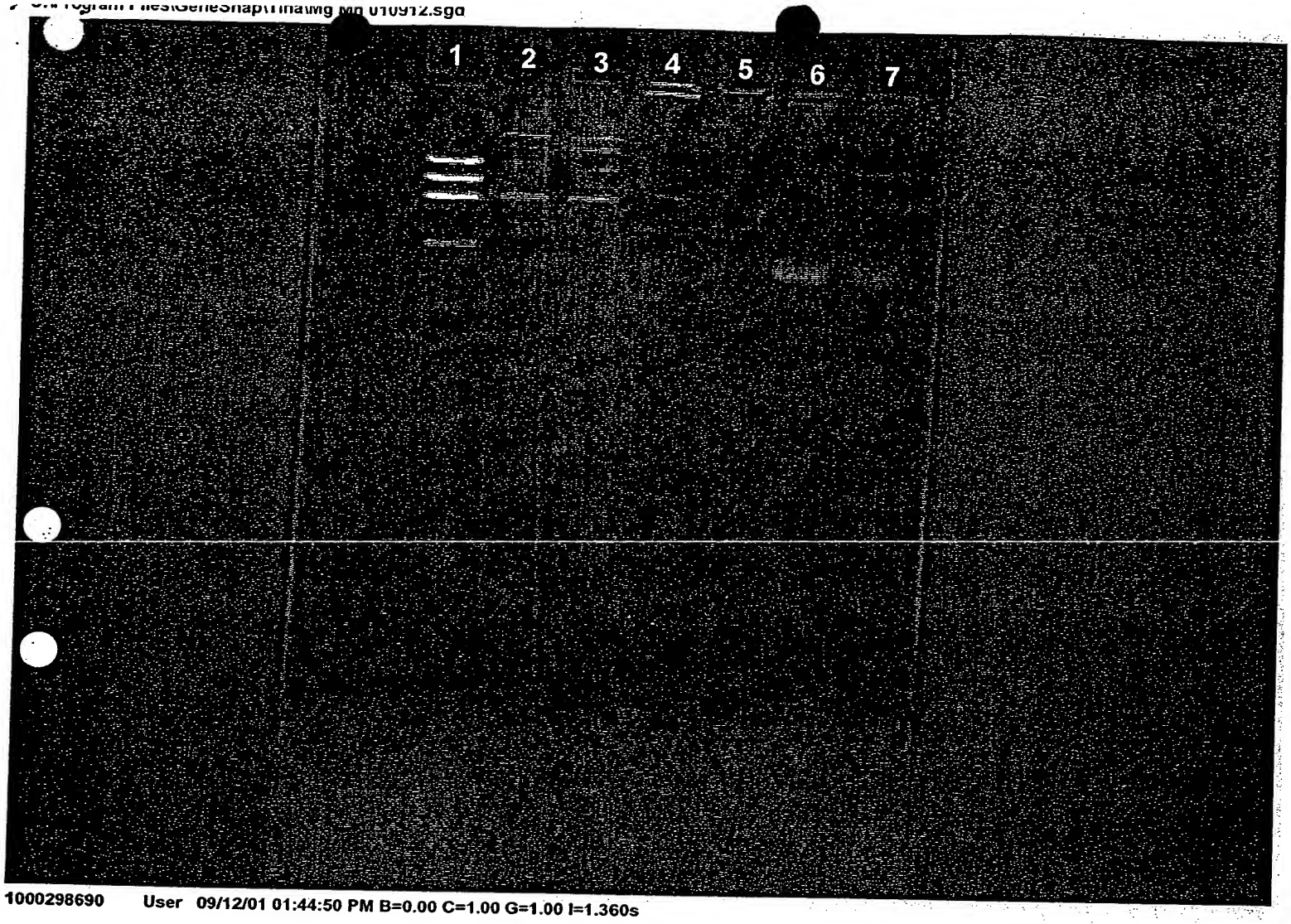
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FIGURE 8

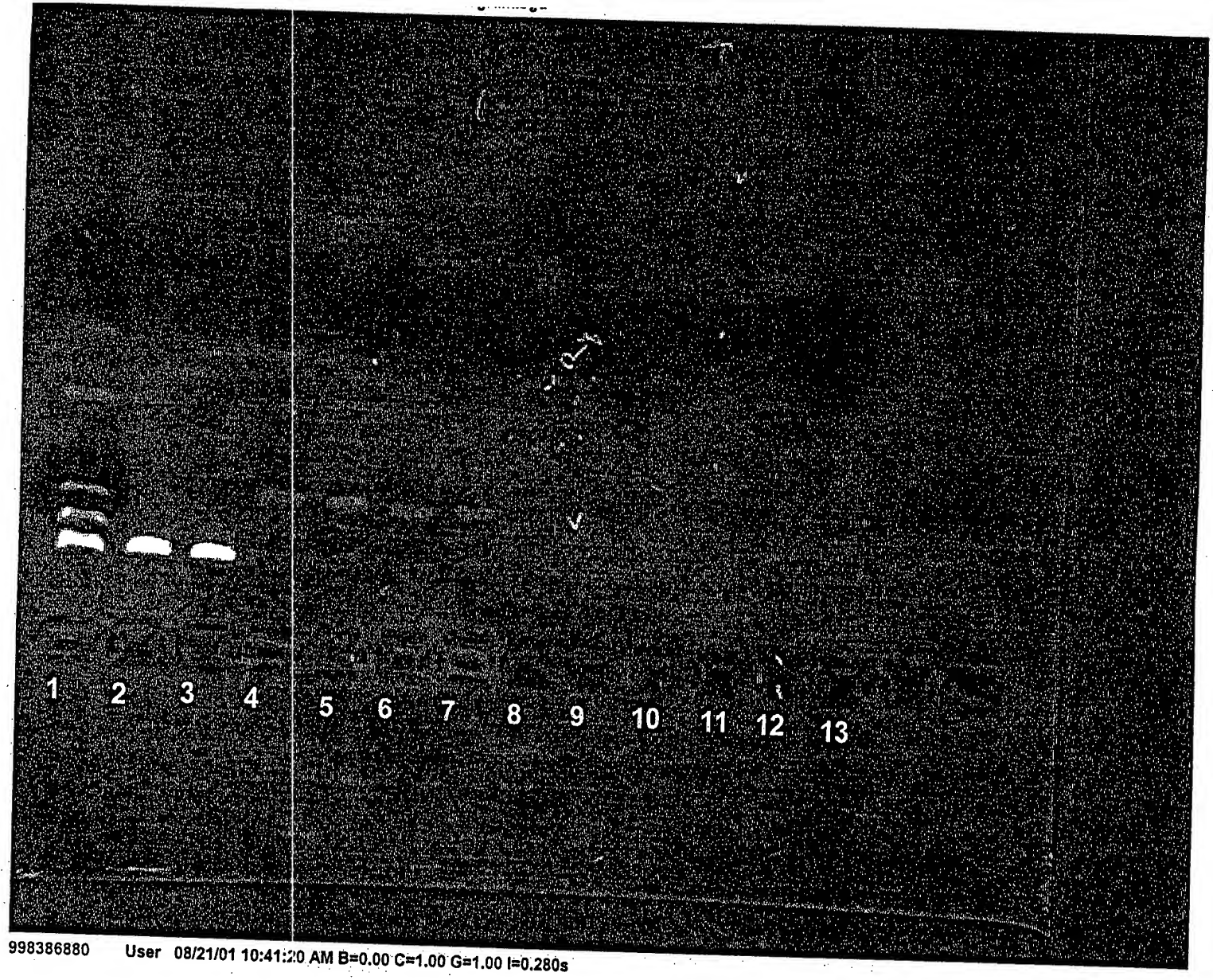
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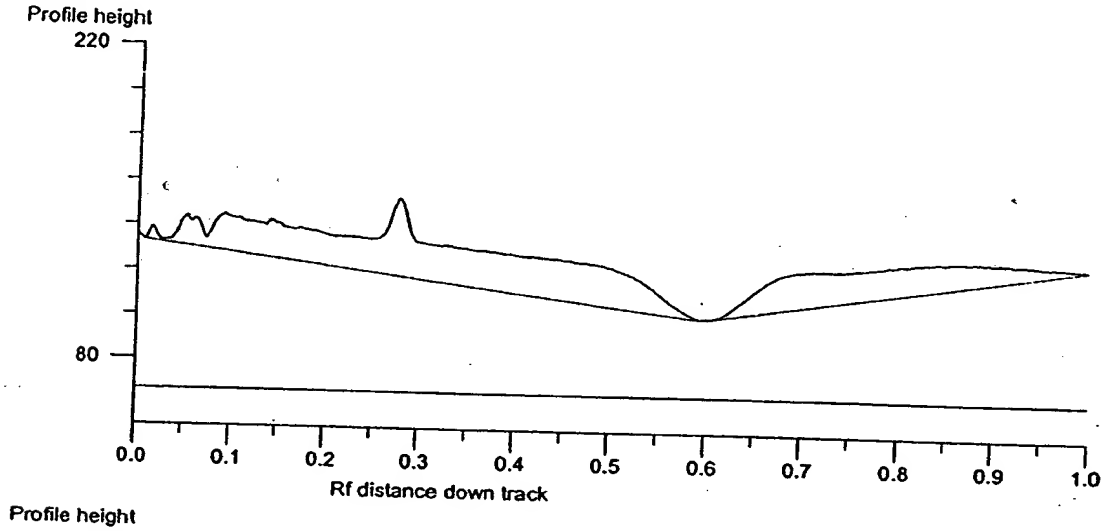
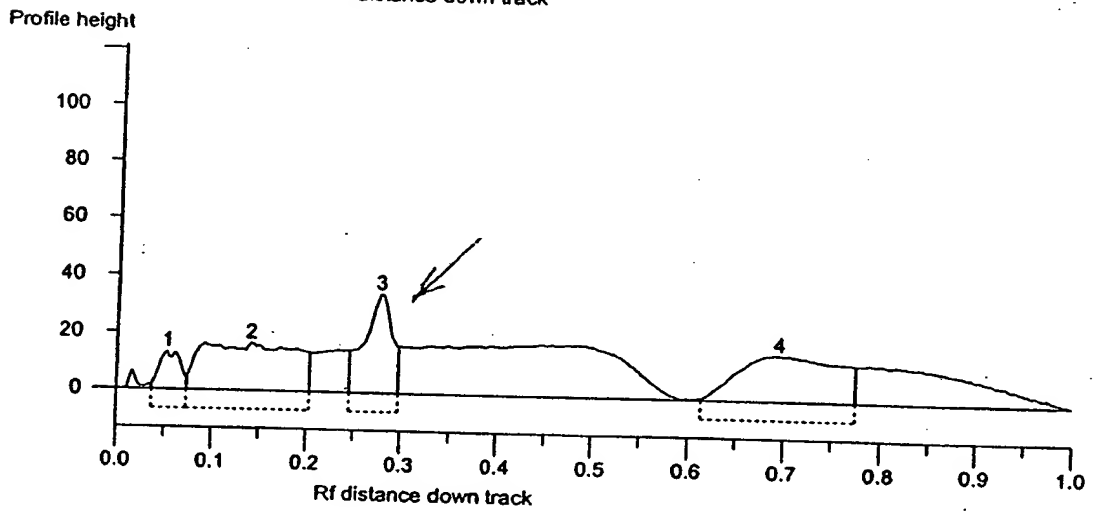
FIGURE 9

Track 6





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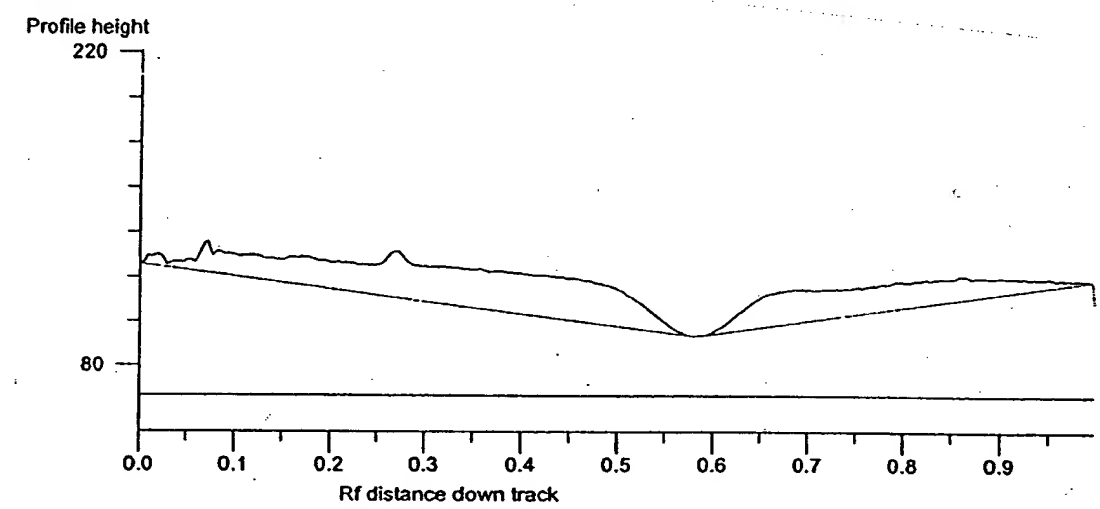
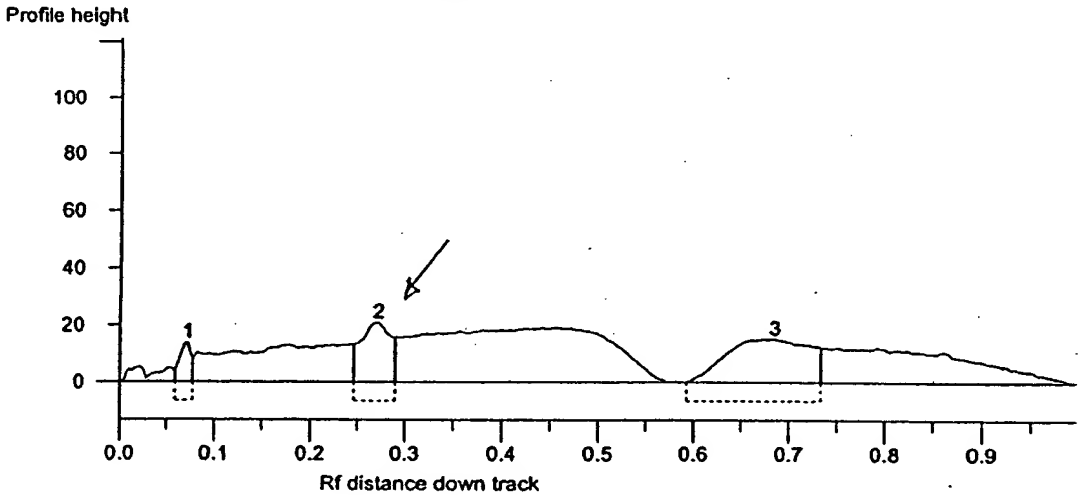
4	0.00	16.002	31320.35

FIGURE 10

Track 12





Track 12			
Number	Mol. weight	Height	Raw vol.
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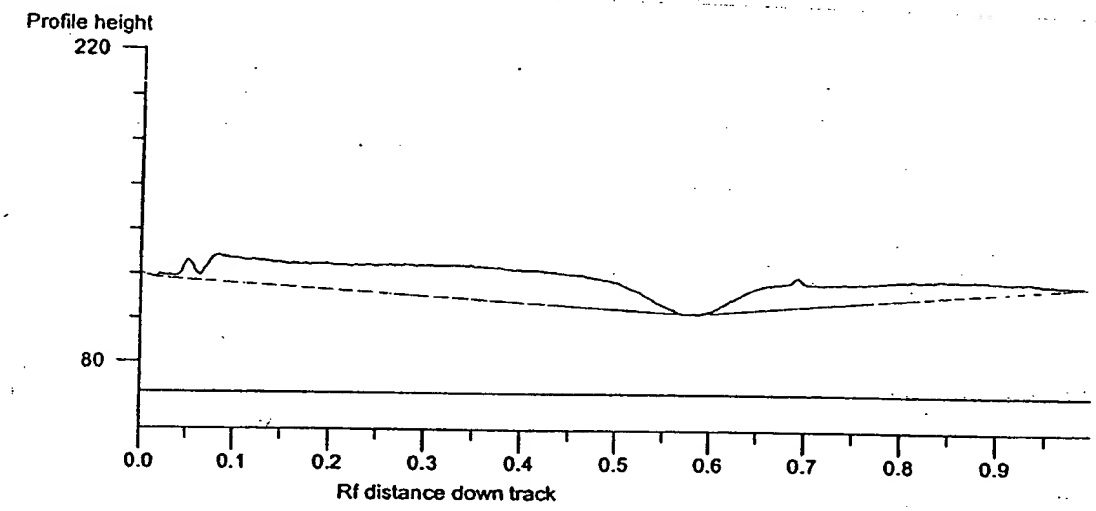
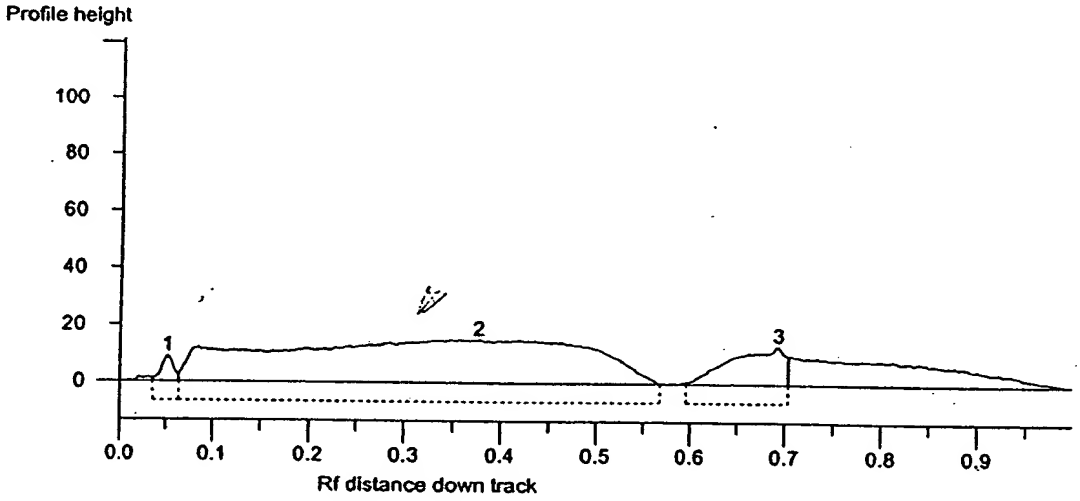
FIGURE 11

Track 13





Track 13			
Number	Mol. weight	Height	Raw vol.
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2	0.00	15.183	97844.64
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FIGURE 12

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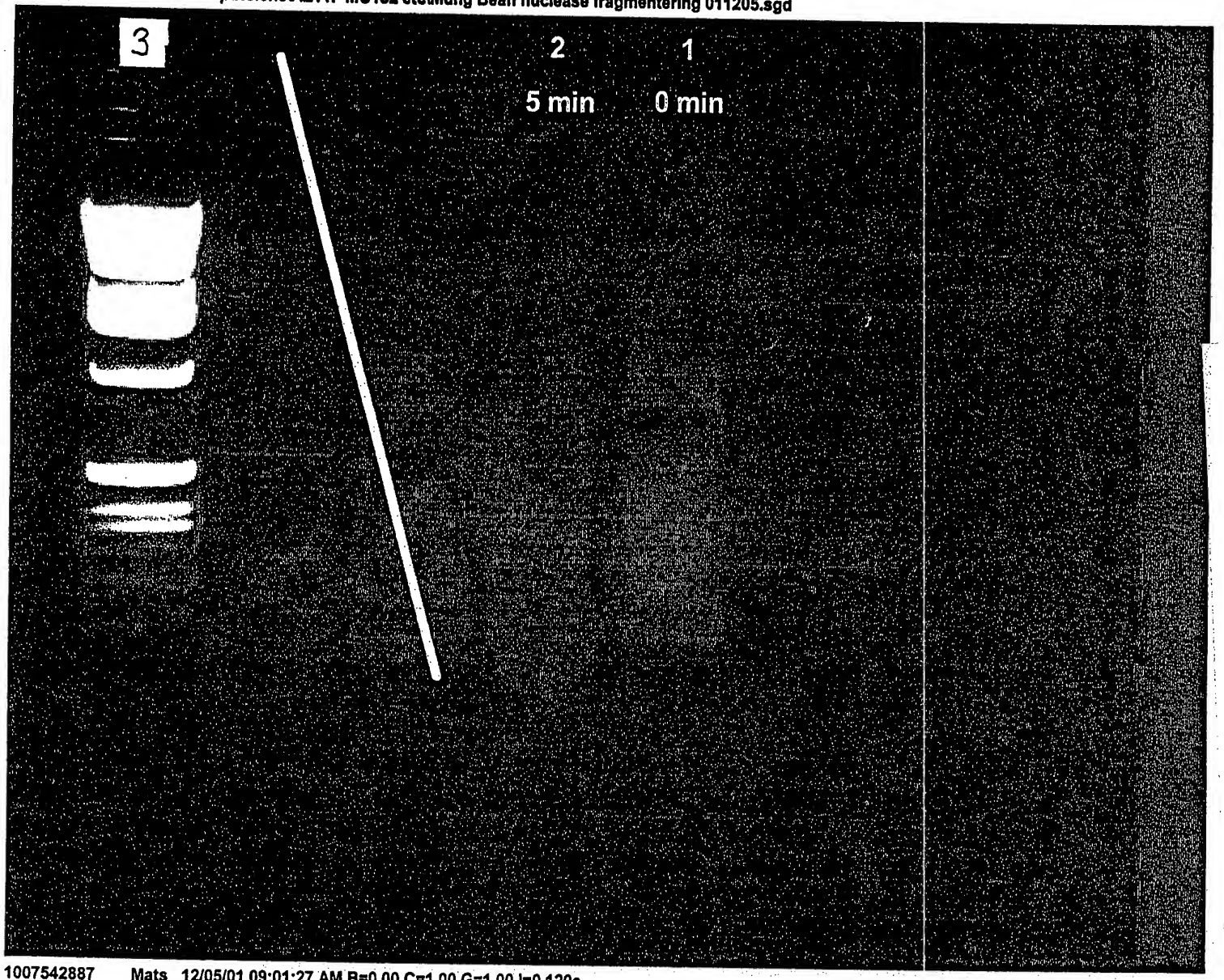

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FIGURE 13